Effect of Ser/Thr Phosphatases on the Recycling of Metabotropic Glutamate Receptor 5 (mGluR5) in HEK293 cells

Bia Roy

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Certificate of Examination

This is to certify that the dissertation titled "Effect of Ser/Thr Phosphatase Inhibitory Drugs on Recycling of Metabotropic Glutamate Receptor 5 (mGluR5) in HEK293 cells." submitted by Ms. Bia Roy (Reg. No. MS09037) for the partial fulfilment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

Dr. Kausik Chattopadhyay Dr. Arunika Mukhopadhaya Dr. Samarjit Bhattacharyya
(Supervisor)

Dated: April 25, 2014

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Dr. Samarjit Bhattacharyya at the Indian Institute of Science Education and Research Mohali. This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Bia Roy

(Candidate)

Dated: April 25, 2014

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr. Samarjit Bhattacharyya

(Supervisor)

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List of figures

Abbreviations

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
CaM	Calmodulin
CaMKII	Calmodulin Kinase II
CNS	Central nervous system
cAMP	cyclic AMP
DAG	Diacylglycerol
DMEM	Dulbecco's modified eagle's medium
EDTA	Ethylene diamine tetraacedic acid
FBS	Fetal bovine serum
GPCR	G-Protein Coupled Receptor
GRK	G-protein coupled receptor kinase
GTP	Guanosine-5'-triphosphate
HEK293 cells	Human embryonic kidney 293 cells
IP3	Inositol 1,4,5-triphosphate
LTD	Long term depression
LTP	Long term potentiation
NGS	Normal goat serum
NMDA	N-methyl-D- aspartate
PBS	Phosphate buffer saline
PKC	Protein kinase C
PFA	Paraformaldehyde
PKA	Protein kinase A
PLC	Phospholipase C
PNS	Peripheral nervous system
PP	Protein phosphatase
PP1	Protein phosphatase 1
PP2A	Protein phosphatase 2A
PP2B	Protein phosphatase 2B
RGS	Regulators of G protein signaling

Abstract

Metabotropic Glutamate receptors are G- Protein coupled receptors for L-glutamate, an excitatory neurotransmitter. mGluR5 is a subtype of Group I mGluRs which plays a critical role in neuronal development, activity dependent synaptic plasticity and implicated in various neuropsychiatric disorders. mGluR5 undergoes endocytosis via two pathways- ligand-mediated and ligand independent or constitutive pathway. Previous study has shown that the mGluR5 receptors undergoing constitutive endocytosis, enter the recycling compartment and recycle back to the cell surface in HEK293 cells. Here I have studied the effect of serine/threonine protein phosphatase inhibitory drugs in the recycling process after constitutive endocytosis of mGluR5 to investigate the role of protein phosphatases, if any, in this pathway.

Contents

Chapter I

Introduction

1.1 Glutamate receptors:

Glutamate is a predominant excitatory neurotransmitter in the central nervous system. It regulates cellular and synaptic activity, neuronal development, neuronal cell death and survival, excitotoxicity, learning and memory, pain perception, and motor activity(1-7). Glutamate is synthesized in the neurons from local precursors, packed into synaptic vesicles and released from the presynaptic sites.

There are two types of glutamate receptors present at the postsynaptic sites as follows:

1. Ionotropic receptors (ligand-gated cation channels):

On the basis of agonist preference these are subdivided as (1) :

- N-methyl-D-aspartate(NMDA),
- α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA); and
- Kainate receptors.

2. Metabotropic glutamate receptors (receptors coupled to G-proteins)

1.2 Metabotropic Glutamate Receptors (mGluRs):

Metabotropic glutamate receptors belong to the G protein-coupled receptor (GPCR) superfamily consisting of twelve members. These are encoded by multiple alternative splicing products of eight distinct genes which in mammals share 30 – 60% identity. On the basis of sequence similarity, pharmacology and second messenger pathway activated upon ligand binding, these are divided into three subgroups (1-5):

- Group I (mGluR1 and mGluR5),
- Group II (mGluR2 and mGluR3) and
- Group III (mGluR4, mGluR6, mGluR7, and mGluR8).

Group I mGluRs activate the G_q proteins coupled to them and the G-proteins initiates Phospholipase C /Inositol 1,4,5-triphosphate /diacylglycerol (PLC/IP3/DAG) signaling cascade. Groups II and III both negatively regulate adenylyl cyclase pathway by reducing

cAMP levels by signaling through the inhibitory G-protein, G_i (8,9).

The basic structure of the mGluRs consists of the amino terminal domain (ATD) followed by the cysteine rich domain (CRD), the seven transmembrane domains (TMD; numbered) and the C-terminal domain (CTD). Upon Glu binding, ATD undergoes a conformational change causing formation of disulfide bridges in cysteine residues of the CRD and activates the bound G-proteins (10, 11). The different types of mGluRs vary in the CTD regions. Some isoforms of the Group I mGluRs (mGluR1a and 5a) contain a very long proline-rich domain also called the Homer1 binding motif that facilitates coupling of mGluRs with other proteins like NMDA receptors.

mGluRs are localized throughout the Central nervous system (CNS) and Peripheral nervous system(PNS). Group I receptors are generally found on postsynaptic cells while groups II and III receptors are generally found on presynaptic cells in the nervous system (12,13) however all three groups of receptors have been found on both presynaptic and post synaptic cells as well as in glial cells(15, 16). The mGluRs have their downstream targets in membrane as well as cytoplasm and nucleus thereby initiation of diverse responses (16, 17, 18). Other than neural cells, mGluRs are also found in major organ systems like epithelium of gastrointestinal system, genitourinary tract, bone, muscle, immune system and hepatosplenic system (14). Studies have shown that mGluRs also play significant functional role in organ homeostasis (9).

1.3 Group I mGluRs:

The group I mGluRs consists of mGluR1 and mGluR5. mGluR1 is mostly distributed in the CA3 pyramidal cells of the hippocampus, Purkinje cells of the cerebellum, the olfactory bulb and thalamus. mGluR5, on the other hand is found in low level in the cerebellum, but abundantly in the cortex striatum, nucleus accumbens of the basal ganglia, CA1 and CA3 pyramidal cells of the hippocampus and granule cells of the olfactory bulb (19). Outside the brain, group I mGluRs are found out in unmyelinated sensory afferent terminals in the skin

and take part in pain sensation (20), melanocytes (21), osteoblasts (22), heart cells (23), and hepatocytes (24). Although Group I mGluRs are present in both presynaptic and postsynaptic cells in the brain, they are primarily localized at peri-synaptic region of the postsynaptic membrane of glutamatergic neurons (25, 26, 27).

Activation of group I mGluRs can lead to activation of a number of signaling pathways:

- Activation of $G_{\alpha\ q/11}$ proteins stimulates PLCβ facilitating the formation of IP3 and DAG from PIP2.(8,9,16)
- Facilitation of L-type voltage dependent Ca^{2+} channels (L-VDCCs) that induces Ca^{2+} influx through L-VDCCs (28,29) from intracellular stores. PKC has been found to activate phospholipase D (PLD), phospholipase A2 (PLA2), and regulate other ion channels.(8,9).
- Activation of $G_{\alpha s}$ and $G_{\alpha i/o}$,and other molecules independent of G-protein (9,15,18).
- Activated group I mGluRs can activate a wide range of protein kinase pathway depending on the population of neurons including cAMP dependent protein kinase (PKA), Ca^{2+} calmodulin dependent protein kinases (CaMKs), mitogen-activated protein kinases (MAPKs), phosphoinositide 3-kinase (PI3K), mammalian target of rapamycin (mTOR), p70 S6 kinase, casein kinase 1, and cyclin-dependent protein kinase 5 (9,13,16,30,31,32,33).

Group I mGluRs play an important role in the two classical forms of synaptic plasticity-long-term potentiation(LTP) and long-term depression(LTD) which represent the cellular mechanisms for learning and memory formation.

1.4 Regulation of group I mGluRs:

GPCRs can be regulated at the level of the G protein or at the level of the receptors. The RGS (Regulators of G protein signaling) family of proteins acts at the G protein level by specifically interacting with the G protein α subunits and accelerating its GTPase activity which in turn attenuates the GPCR activity (34, 35). On the other hand, at the level of GPCRs, their activity is regulated by desensitization upon agonist binding that acts as a feedback mechanism to protect against both acute and chronic receptor over-stimulation.

This involves phosphorylation, arrestin binding and internalization (36, 37).

1.4. 1. Regulation of G Proteins

RGS2 and RGS4 have been shown to inhibit Group I mGluR activity by specifically interacting with $G_{\alpha\ q/11}$ -like G proteins and blocking the subsequent activation of PLC in recombinant and native systems (38, 39). In Xenopus oocytes, purified RGS4 attenuates the mGluR1a- and mGluR5a- mediated activation of a calcium dependent chloride current (38). It also attenuates the mGluR5-mediated dose-dependent inhibition of potassium currents in hippocampal CA1 neurons.

1.4. 2. Desensitization

The canonical model for GPCR desensitization involves G protein-coupled receptor kinase (GRK)-mediated phosphorylation followed by β -arrestin binding that leads to uncoupling of the GPCR from the G protein. However, Group I mGluRs undergo both agonist-mediated (homologous) and agonist independent (heterologous) desentisization. This can occur via second messenger-dependent protein kinases (40, 41), calmodulin kinase II (42), GRK (43, 44), and the huntingtin-binding protein optineurin (45).

1.4. 3. Endocytosis

1.4.3a. Agonist-mediated endocytosis

Group I mGluRs internalize via multiple mechanisms depending on the trigger for internalization and the splice variant. Upon agonist activation Group I mGluRs undergo rapid internalization. This is strongly inhibited by the expression of both β -arrestin- and dynamin dominant-negative mutants (62, 63). Glutamate activation of mGluR1a has shown to increase GRK2/ β -arrestin association with mGluR1a (41) thereby promoting the membrane translocation of both β -arrestin1 and β -arrestin2 from cytosol to membrane, leading to mGluR1a internalization (63). The agonist-stimulated endocytosis of mGluR1a and mGluR1c is partly regulated by PKC as inhibitor of PKC blocks internalization of these receptors by 50% in HEK293 cells (42)

Among heterologous $G_{\alpha s}$ and $G_{\alpha q/11}$ -coupled receptors, Agonist-activation of m1 muscarinic acetylcholine or P2Y1 purinergic receptors can also trigger the GRK2-, PKC/CaMKII-, β-arrestin and clathrin-dependent internalization of mGluR1a (42, 43). However, PKA activation inhibits both homologous and heterologous internalization of mGluR1a and mGluR1b probably via inhibiting the association of GRK2/β -arrestin with mGluR1a and mGluR1b.

1.4.3b. Constitutive endocytosis

Group I mGluRs also undergo constitutive endocytosis in neurons as well as heterologous systems like HEK293 cells. To verify that this internalizations is not a consequence of release of glutamate from cells, they were treated with antagonists and released glutamate was metabolized. This had no effect on the loss of surface receptors(62,64). While overexpression of GRK2 reduced the agonist-mediated endocytosis of mGluR1a, it did not have any effect on constitutive endocytosis suggesting that constitutive endocytosis of mGluR1a is GRK-mediated phosphorylation independent (62,64). In case of treatment with inverse agonist, while constitutive endocytosis of mGluR1a was blocked(65), constitutive endocytosis of mGluR5a remained unaffected(66). The role of β -arrestins in constitutive endocytosis is controversial. While one study showed that the constitutive endocytosis of mGluR1a is although mediated by clathrin-coated vesicles, it is β -arrestin and dynamin independent(62, 64). Another study showed that the same process is both β -arrestin and clathrin dependent(65). This may be explained as mGluR can form a complex with RalGDS which can function to recruit β -arrestins. However the constitutive internalization of mGluR5a is shown to be clathrin-independent(66) although constitutively internalized mGluR1a and mGluR5a both colocalize with clathrin in HEK293 cells and that constitutively internalized mGluR1a and mGluR5a are colocalized in the same endocytic vesicles (62, 64, 67). This can be explained as either mGluRs use different mechanisms for endocytosis or functionally distinct population of clathrin coated vesicles exist that mediated the internalization of receptors to the same endocytic compartment(68). Constitutive endocytosis of mGluR1a and mGluR5a was shown to be dependent on Ral/PLD2/RalGDS containing scaffolding protein complex in heterologous cell systems and primary cortical neurons(67).

Since β -arrestin is shown to form a complex with RalGDS that leads to the stimulation of actin cytoskeletal rearrangement (69), there may be a cross talk between the agonist-mediated and agonist independent pathway of internalization of mGluRs.

1.5 Phosphorylation of Group I mGluRs:

1.5a PKC mediated phosphorylation

mGluR1a expressed stably in heterologous cells showed significant constitutive phosphorylation(46). Also agonist-mediated activation of mGluR1a induced a rapid and transient increase in the phosphorylation level and this was shown to be abolished by a specific PKC inhibitor Ro318220 (46) and use of a PKC activator mimicked the effect of the mGluR1/5 agonist in phosphorylating mGluR1c (47) suggesting the role of PKC in phosphorylation of Group I mGluRs. A PKC-mediated phosphorylation site has been identified in rat mGluR5a in heterologous cells: a threonine residue at position 840 (T840) within the proximal region of mGluR5a C-terminal tail (48). However, studies suggest the possibility of existence of another phosphorylation site. CaM (calmodulin), a Ca^{2+} binding protein can regulate PKC-mediated phosphorylation. Activation of both mGluR1 and 5 lead to the release of Ca^{2+} from intracellular stores, however, the pattern of response varies which seems to be regulated by the PKC-mediated phosphorylation. In transfected cells, while mGluR1a activation induced a single-peaked Ca^{2+} rise, mGluR5a activation induced characteristic Ca^{2+} oscillations (48). A recent study suggests that in case of mGluR5a, the serine 839 (S839) is phosphorylation site while T840 has a permissive role in the PKC-dependent phosphorylation of S839 (49).

1.5b GRK mediated phosphorylation

GRK2, GRK4, and GRK5 play a role in mGluR1 desensitization in heterologous or Purkinje cells (43,54, 56). The co-expression of GRK2 (43) or GRK4 (54) in HEK293 cells showed enhanced phosphorylation of mGluR1a in constitutive and/or agonist-stimulated processes. However agonist-stimulated mGluR1a phosphorylation was reduced by GRK2 dominant-negative mutants (43). Another study shows that GRK2 mediated the phosphorylation of serine/ threonine sites within the last 150–333 amino acids of mGluR1a C-terminus(55).

PKC and CaMKII facilitate the association of GRK2 with mGluR1a thus promoting internalization and desensitization of mGluR1a upon agonist-activation(42). GRK2 can also regulate mGluR5 in kinase activity-dependent manner(57) and T840 site in mGluR5 has been implicated to be partially important in this process.

1.5c CaMKII mediated phosphorylation

CaMKII is highly expressed in the synaptic regions of brain cells(52). It is activated upon the binding of Ca^{2+} and CaM which then phosphorylates exogenous substrates as well as its autophosphorylation site (T286 in the α isoform). Based on sequence analysis, mGluR1/5 has multiple sites predicted for a phosphorylation substrate consensus, RXX(S/T) which are present in the C-terminus region of mGluR1a and mGluR5a (53). CaMKII and mGluR1/5 are found to be co-clustered at the defined PSD microdomain, thereby suggesting that mGluR1 and/or 5can be a substrate of CaMKII. Pharmacological studies indicate that CaMKII actively regulates mGluR1a upon glutamate stimulation and this is required for the internalization and homologous desensitization of mGluR1a (41). There might be significant cross-talk between CaMKII, conventional PKC (α, β, γ),and CaM since they bind to the intracellular region of group I mGluRs.

1.5d Tyrosine mediated phosphorylation

The protein tyrosine kinase inhibitors inhibited the mGluR1/5 response to the agonist DHPG in midbrain dopamine neurons (50). Tyrosine phosphatase inhibitor pervanadate, enhanced Tyrosine phosphorylation of mGluR5 suggesting the presence of an active endogenous cycle of phosphorylation and dephosphorylation. Another study showed the parallel increase of tyrosine phosphorylation and mGluR5-mediated PI hydrolysis (51) suggesting the role of tyrosine phosphorylation in regulating receptor function. NMDA is known to potentiate mGluR5 function through either enhancing tyrosine phosphorylation or inhibiting PKC phosphorylation or both (51). Unlike PKC-mediated phosphorylation, tyrosine phosphorylation is not agonist sensitive (52).

1.6 Regulation of phosphorylation by PPs

Protein kinases and Protein phosphatases interact to regulate the protein phosphorylation processes. Okadaic acid, a serine/threonine phosphatase PP1/2A inhibitor has been shown to prevent the recovery of mGluR5a from desensitization. Coimmunoprecipitation studies showed that PP2A forms complexes with mGluR5 in neurons in vivo (58). NMDA at low (5–15 μM) concentrations showed enhanced mGluR5 responses through activation of a PP, followed by dephosphorylation of PKC phosphorylation sites on mGluR5 (such as S890). However this did not occur in high (50–100 μM) concentrations. Also, cyclosporin-A, a PP2B selective inhibitor blocked the effect of NMDA on mGluR5-mediated responses. The mGluR5 mutant (S890G), that is resistant to PKC-mediated desensitization, was not potentiated by NMDA and hence desensitized. However, the effect of NMDA was insensitive to okadaic acid (59). This suggests that NMDA works via a phosphatase (likely PP2B) which is different from the resensitization causing phosphatase (likely PP1/2A) in mGluR5 (60). Studies show that both recombinant and native forms of PP1γ1 (PP1C1) binds to the C-terminus of long-form group I mGluRs, i.e., mGluR1a, mGluR5a, and mGluR5b (61). Mapping of PP1γ1-interacting domains within mGluR1/5 C termini has revealed 891-KSVSW-895 for mGluR1a and 880-KSVTW-884 for mGluR5a as conserved PP1γ1 recognition motif (61). However which PP isoforms can directly bind to mGluR1/5 is not known.

1.7 Objective of the study

mGluR5 plays a critical role in neuronal development, activity dependent synaptic plasticity and implicated in various neuropsychiatric disorders. However the trafficking of this receptor is not well understood. Previous study from our lab has shown that the mGluR5 receptors undergoing constitutive endocytosis, enter the recycling compartment and recycle back to the cell surface in HEK293 cells. As mentioned before, group I mGluRs happen to be regulated by protein phosphatases which are showed to bind to the C terminal region of long form of group I mGluRs. However which phosphatases are involved in the recycling of the receptor after constitutive endocytosis is not known. Here, we have studied the role of serine/threonine protein phosphatase, if any, in the recycling process after constitutive endocytosis of mGluR5.

Chapter 2

Materials and Methods

2.1 Materials

2.1.a Plasmids and constructs

Two constructs of mGluR5b were used for the study:

- **myc construct:** full length mGluR5b cDNA tagged with the myc at the N-terminus in pRK plasmid.
- **GFP construct:** mGluR5b tagged with GFP at the C-terminus and cloned in pcDNA3.1.

2.2.b Chemicals and reagents

Dulbecco's Modified Eagle medium (DMEM), Penicillin - streptomycin solution, Antibiotic–Antimycotic mix, Lipofectamine, OptiMEM, Distilled Water, Trypsin-EDTA, Fetal bovine serum (FBS) were purchased from Invitrogen. Ampicillin, PFA, poly-D-lysine, Fluoromount TM Aqueous mounting medium were purchased from Sigma, USA. Fine chemicals were obtained from Life technologies (USA), Merck limited (USA). Anti Myc mouse monoclonal antibody was obtained from Abcam. Secondary antibodies viz., goat anti-mouse Alexa-568 and goat anti-mouse Alexa-647 were purchased from Invitrogen (USA). Drugs: Okadaic acid, Cyclosporin, FK506 (purchased from Sigma) and Endothall (purchased from Calbiochem)

2.2.c Tissue culture wares

35 mm dishes, 60 mm dishes, 100 mm dishes, 24 well plates, 15 ml conical tubes, 50 ml conical tubes, 5 and 10 ml serological pipettes and cryotubes were from BD Falcon (USA) and Tarsons (India). Coverslips and glass slides were from fisher scientific (USA).

2.2 Methods

2.2.1 Cell culture and transfection

HEK293 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, antibiotic-antimycotic mix at 37°C, 5% CO_2.

Transfection was carried out at a cell confluency level of 65 -70%. Cells were plated on a 35 mm coverslip coated with 50 μg/ml poly-D-lysine in a 24-well plate and transfected with DNA at a concentration level 2 μg DNA/ 10 μl lipofectamine in 1 ml Opti MEM and added to the cells for 5 hours. Expression was observed and further experiments were carried out 24-36 hours after transfection.

2.2.2 Constitutive endocytosis assay

24 hours after transfection,HEK293 cells were blocked with 2% normal goat serum (NGS) and incubated with primary antibody (anti-myc mouse monoclonal antibody) (1:500) for 1 hour on ice on a shaker at 5rpm. Control cells were immediately fixed with 4% PFA in PBS for 15 min.

For endocytosis the cells were incubated at 37°C for 5 min, 15 min and 30 min, to allow constitutive internalization of the receptors followed by fixation in ice cold PFA (4%) without permeabilization, then washed with PBS.

The surface receptors were labelled using saturating concentration of first secondary antibody, goat anti -mouse Alexa-568 (1:100) for 1 hour at 37°C and washed with PBS. Then cells were permeabilized with 0.1% Triton X-100 for 30 min at 37°C . After that cells were washed and blocked with 2% NGS in PBS for 1 hour at 37°C followed by second secondary antibody, goat anti-mouse Alexa-647 (1:800) staining for 1 hour at 37°C to label the internalized receptors. The coverslips were mounted on glass slides and imaged under the confocal microscope.

2.2.3 Recycling assay

As described in constitutive endocytosis assay, the live transfected HEK293 cells were stained with primary antibody (anti-myc mouse monoclonal antibody). Control cells were fixed with ice cold 4% PFA in PBS for 15 min on ice (without permeabilization) while other cells were incubated at 37°C for 30 min, 2 h, 2.5 h, 3 h and 3.5 h to check whether the constitutively endocytosed receptors recycle back to the plasma membrane.

After respective time points cells were fixed without permeabilization by ice cold 4% PFA for 15 min on ice. The surface receptors were labelled using saturating concentration of first secondary antibody, goat anti -mouse Alexa-568 (1:100) for 1 hour at 37°C and washed with PBS. Then cells were permeabilized with 0.1% Triton X-100 for 30 min at 37°C . After that cells were washed and blocked with 2% NGS in PBS for 1 hour at 37°C followed by second secondary antibody, goat anti-mouse Alexa-647 (1:800) staining for 1 hour at 37°C to label the internalized receptors. The coverslips were mounted on glass slides and imaged under the confocal microscope.

2.2.4 Drug assay

The live transfected HEK293 cells were incubated in plain DMEM containing 100 nM Okadaic acid, 50µM Endothall, 1µM FK506 and 1µM Cyclosporin for half an hour at 37°C and control cells were incubated in plain DMEM. This was followed by blocking with 2% NGS for 5 min in plain DMEM containing the respective drugs and staining with primary antibody (anti-myc mouse monoclonal antibody) (1:500) containing the drugs in their respective concentrations. Control cells were fixed with ice cold 4% PFA in PBS for 15 min on ice (without permeabilization) while other cells were incubated at 37°C for 30 min, 2 h, 2.5 h, 3 h and 3.5 h in plain DMEM containing the respective drugs to check whether the constitutively endocytosed receptors recycle back to the plasma membrane.

After respective time points cells were fixed without permeabilization by ice cold 4% PFA for 15 min on ice. The surface receptors were labelled using saturating concentration of first secondary antibody, goat anti -mouse Alexa-568 (1:100) for 1 hour at 37°C and washed with

PBS. Then cells were permeabilized with 0.1% Triton X-100 for 30 min at 37°C . After that cells were washed and blocked with 2% NGS in PBS for 1 hour at 37°C followed by second secondary antibody, goat anti-mouse Alexa 647 (1:800) staining for 1 hour at 37°C to label the internalized receptors. The coverslips were mounted on glass slides and imaged under the confocal microscope.

2.2.5 Confocal microscopy

Images were captured using a 63X oil immersion objective in Zeiss LSM 780 laser scanning confocal microscope. Series of optical sections (3-5) were collected at step size of 0.43 μm. Digital offset, gain and laser power for each channel were set and kept constant for individual experiment. Alexa 568 and Alexa 647 excitation/emission were achieved using appropriate filter sets.

2.2.6 Image Analysis

Image J software was used for analyzing all images. All the optical stacks of each image were first merged using z project option for maximum intensity projection. Next the thresholding values for each fluorophore were set and kept constant for each individual experiment and thresholded area was measured.

The internalization index was measured by normalizing the internalized receptor with the total (surface + internal) receptors. For representation every image of an individual experiment was adjusted using identical brightness and contrast.

Chapter 3

Results

3.1 Constitutive endocytosis of myc-mGluR5b in HEK293 cells

GPCRs undergo both ligand-mediated endocytosis and constitutive endocytosis. Upon ligand activation, mGluR5 activate the subsequent G-protein signaling pathway leading to the formation of IP3 and DAG from PIP2 which subsequently increases the intracellular Ca^{2+} levels. However a small fraction of mGluR5 also undergoes constitutive endocytosis to maintain the basal level of receptor expression. To study the constitutive endocytosis of mGluR5, we have used myc-tagged mGluR5 in this study(Fig. 1). HEK293 cells were transfected with myc-mGluR5b and 24 hours after transfection live cell antibody staining assay was performed to visualize and quantitate the fraction of internalization. All the surface receptors were subjected to primary antibody staining on ice such that no intracellular trafficking takes place and only the surface receptors are bound to the primary antibody. After allowing constitutive endocytosis during incubation at 37°C for 5 min, 15 min and 30 min, the cells were fixed. Control cells were fixed immediately. To visualize the surface receptors, first secondary tagged with Alexa 568 fluorophore was used in saturating concentration. The internalized receptors were labelled after permeabilizing the cells with triton X-100 by second secondary antibody tagged with Alexa 647 fluorophore. The result suggests that the myc-mGluR5 internalized constitutively as seen by the increase in the intracellular fluorescence over time (Fig. 2A). Quantitation also showed values for average fold increase in fluorescence at 5 min (1.07± 0.200), 15 min (1.52±0.311) and maximum internalization in 30 min (1.87±0.381) (Fig. 2B).

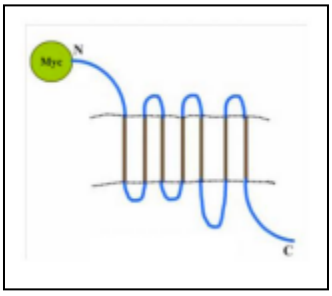
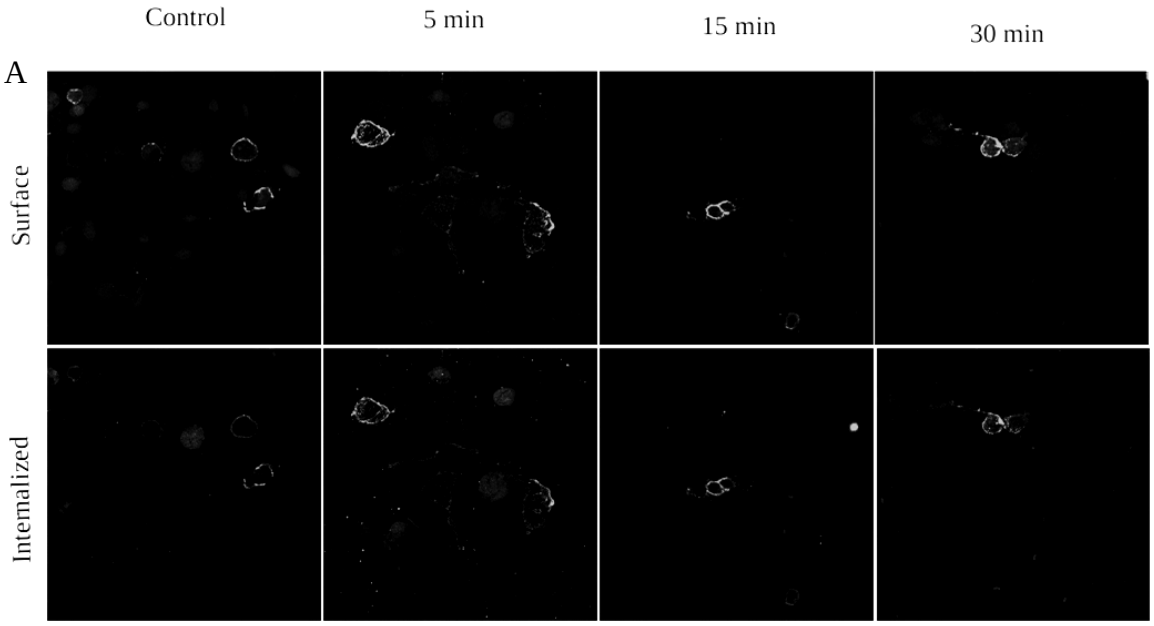


Figure 1. myc-mGluR5 construct

Myc-mGluR5 construct is shown in the picture. Myc is tagged at the N-terminus of mGluR5b.



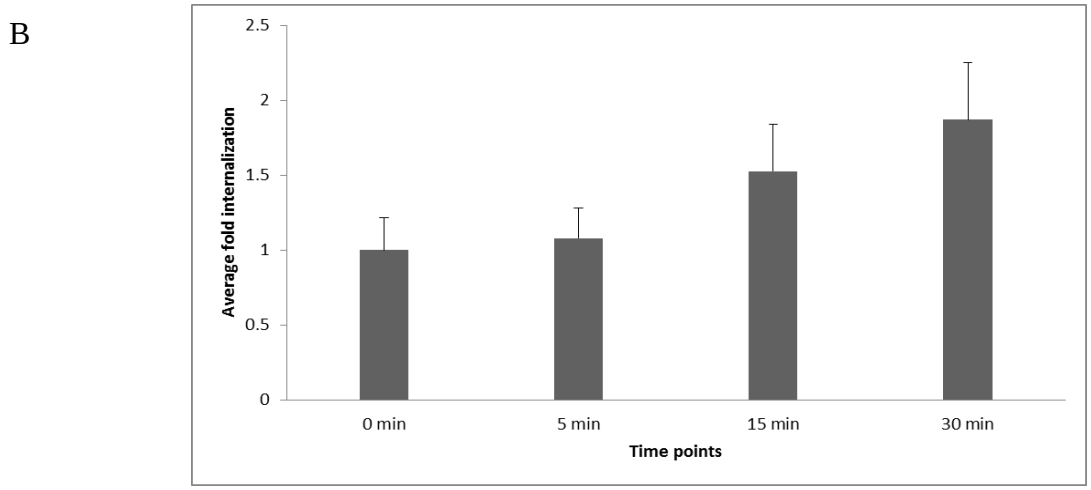


Figure 2. Constitutive endocytosis of myc-mGluR5b in HEK 293 cells

A. The intensity of internal fluorescence increases as the time period of incubation increases and shows maximum at 30 min while 5 min shows internal fluorescence almost close to the control cells. B. Quantitation also shows while 0 min and 5 min has almost equal internal fluorescence it increases

in 15 min and reaches maximum in 30 min.

3.2 Kinetics of recycling of constitutively endocytosed mGluR5b

GPCRs undergo rapid desensitization after ligand activation mostly by phosphorylation and arrestin binding and undergo endocytosis. While some GPCRs activate signaling cascades at the surface prior to desensitization, some GPCRs continue to signal even from within the intracellular compartments. After internalization into endosomes, GPCRs can follow either recycling pathway by getting sorted into the recycling endosome and undergo dephosphorylation to reach back to the plasma membrane in resensitized state. Some GPCRs can also follow the degradative pathway by getting sorted into lysosomal compartment and hence terminate the signal (downregulation). mGluR5 undergoes recycling subsequent to both ligand-mediated (unpublished observation) and constitutive endocytosis. To study the recycling of mGluR5, constitutive endocytosis assay was carried out in HEK293 cells transfected with myc-mGluR5b DNA as described before with incubation time 30 min, 2 hr, 2.5 hr, 3 hr and 3.5 hr at 37°C followed by the primary antibody staining. Cells showed an increase in internal fluorescence till 30 min followed by gradual decrease of the internal fluorescence in subsequent time points (Fig. 3A). Quantitation also showed a peak in internal fluorescence intensity at 30 min (1.74±0.096) followed by gradual decrease of internal fluorescence at 2 hr (1.51±0.105), 2.5 hr (1.24±0.113), 3 hr (1.18±0.100), and almost close to control level at 3.5hr(1.10±0.216) (Fig. 3B). Data from our lab earlier showed that the receptor enters the recycling compartment subsequent to constitutuive endocytosis and no detectable localization of the receptor was observed in the lysosome (70). All these data suggest that mGluR5 recycles to the cell surface subsequent to the constitutive endocytosis.

3.3 Effect of protein phosphatase inhibitors on recycling of constitutively endocytosed mGluR5b

Phosphorylation status of a protein depends on the fine tuning between activity of kinases and phosphatases. mGluR5a has been shown to be associated with protein phosphatase PP2A. Okadaic acid, a PP1/2A inhibitor has been shown to prevent the recovery of mGluR5a

from desensitization while cyclosporin-A, a PP2B selective inhibitor blocked the effect of NMDA on mGluR5-mediated responses (58,59). Hence, here we wanted to investigate the role of PP1/PP2A/PP2B on the recycling of constitutively endocytosed mGluR5b, if any, in HEK293 cells. Thus the HEK293 cells after transfection with myc-mGluR5b, were pre-incubated in plain DMEM containing Okadaic acid, Endothall, FK506 and Cyclosporin in their respective concentrations. The subsequent steps of blocking, primary antibody staining and incubation to allow endocytosis were carried out in plain DMEM containing the respective drugs. This was followed by secondary antibody staining as described before. Cells showed contradictory results in two different sets of experiments.

A

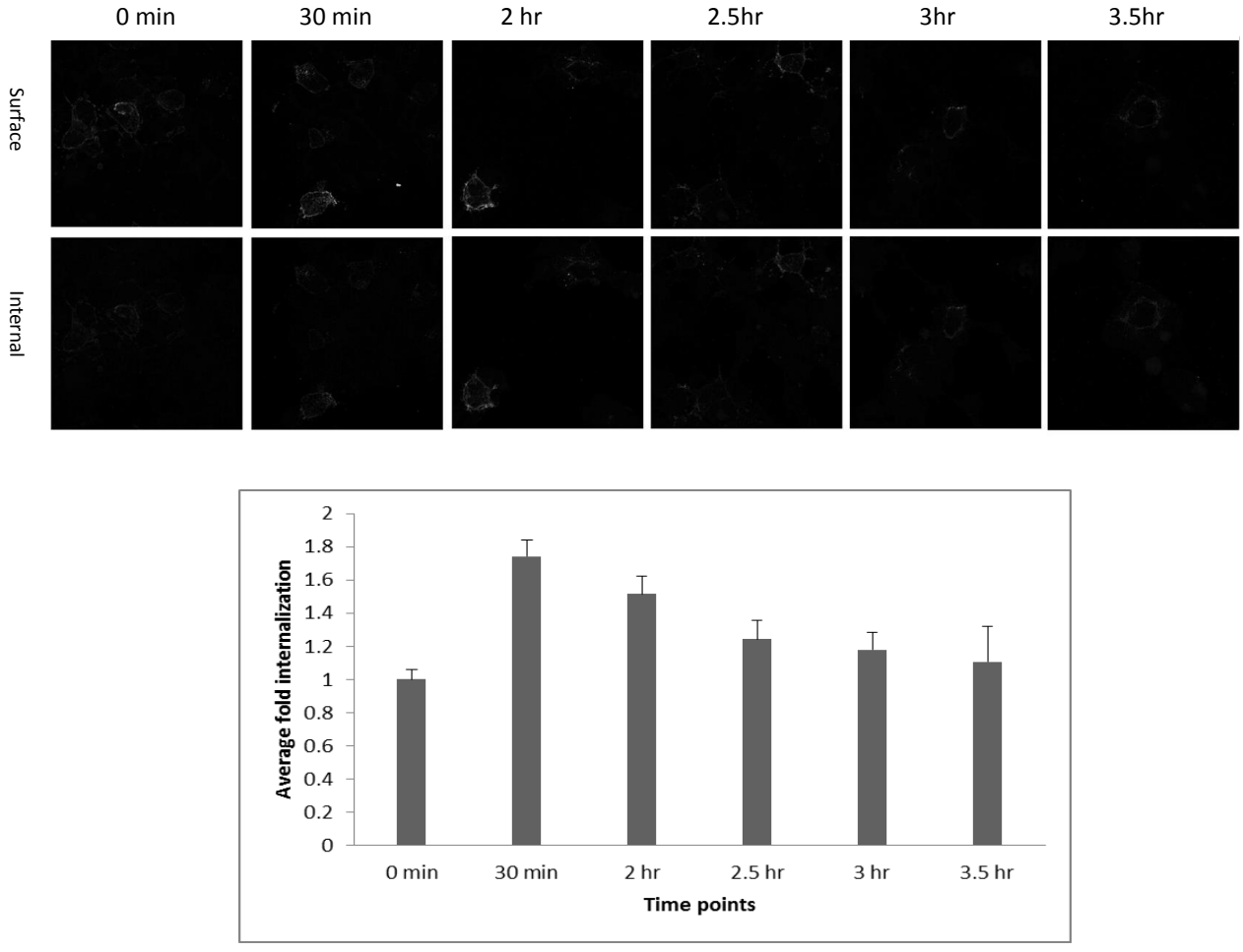


Figure 3. Recycling of constitutively endocytosed mGluR5b receptors in HEK 293 cells

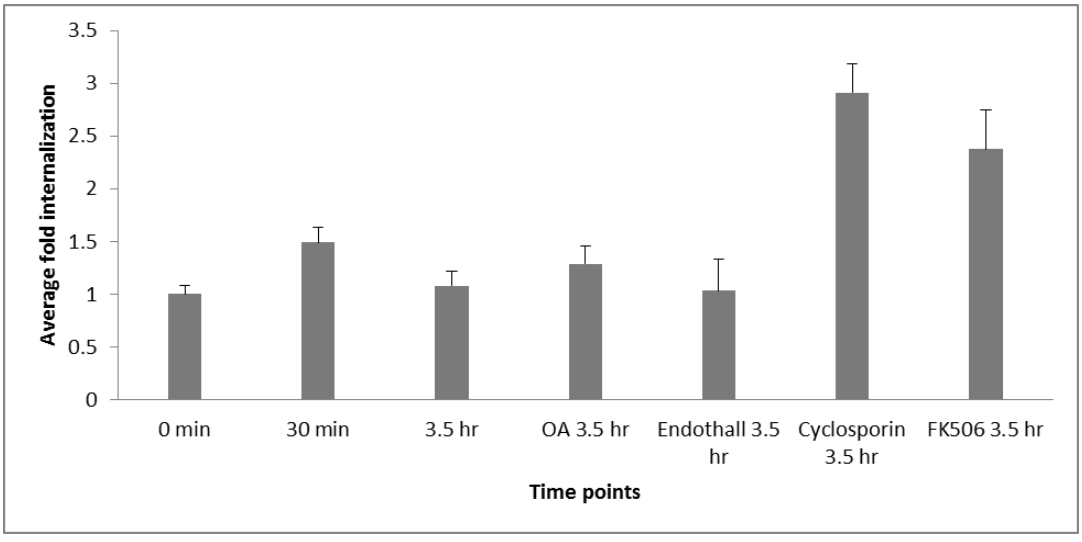
A. The intensity of internal fluorescence increased to maximum till 30 min incubation but decreased

in subsequent time points suggesting that the receptors recycle and come back to the surface at 3.5 hr. B. Quantitation also showed increase in internal fluorescence intensity at 30 min and subsequent decrease at latter time points reaching close to control at 3.5 hr.

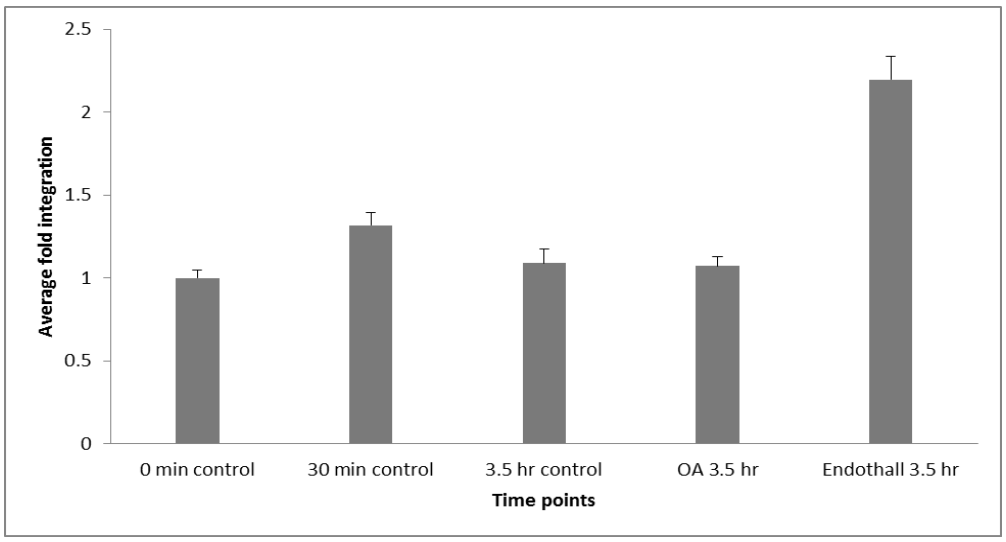
First experiment showed that at 3.5 hour cells treated with okadaic acid had internal fluorescence reduced almost close to control cells (1.28±0.167) also endothall treated cells showed similar internal fluorescence (1.03±0.295) suggesting no effect of PP1/PP2A inhibitors on the recycling process subsequent to constitutive endocytosis (Fig. 4A). However second experiment showed in 3.5 hr cells treated with okadaic acid had internal fluorescence (1.07±0.059) similar to control cells (1.08±0.083) while endothall treated cells had higher internal fluorescence (2.19±0.143) than control cells at 30 min (1.31±0.075) although similar to the cells treated with endothall at 30 min (2.18±0.171) (Fig. 4B) . This result is inconclusive since both the inhibitors work on the same group of phosphatases.

For PP2B selective inhibitors, the first experiment showed internal fluorescence higher than control cells at 3.5 hr, for cyclosporin (2.90±0.278) and FK506 (2.37±0.369) in 3.5hr. The second experiment showed while the internal fluorescence of FK506 treated cells at 3.5 hr (1.68±0.066) was comparable to control cells at 3.5 hr (1.60±0.081), the internal fluorescence for cyclosporin treated cells at 3.5 hr (2.21±0.117) was comparable to control cells at 30 min (2.36±0.112) (Fig.4C). This result is also contradictory since FK506 and cyclosporin are both PP2B selective inhibitors they are supposed to show similar effects. Also the control cells in 3.5 hr did not show complete recycling of receptors.

A



B.



C.

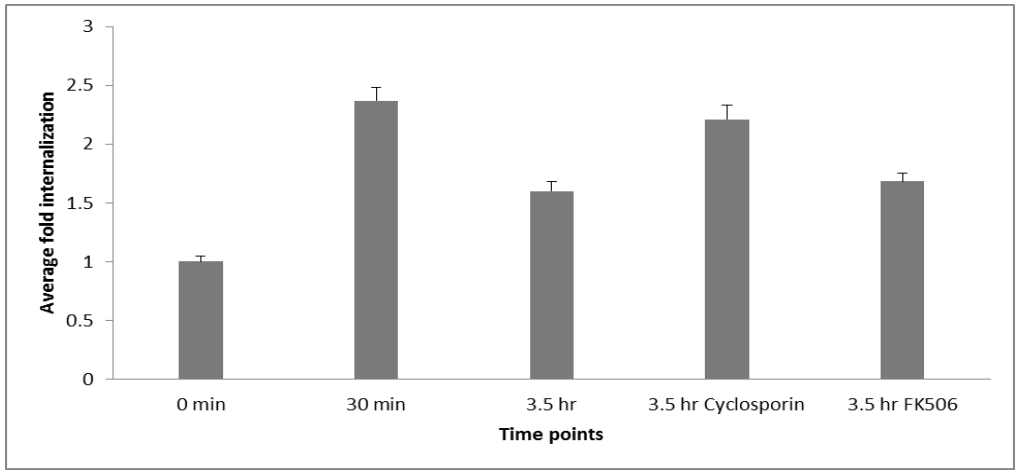


Figure 4. Effect of phosphatase inhibitors on recycling of constitutively endocytosed mGluR5b receptors in HEK 293 cells

A While okadaic acid and endothall treated cells showed internalized fluorescence at 3.5 hr comparable to control cells at 0 min, cyclosporine and FK506 treated cells showed higher internalized fluorescence at 3.5 hr. B. While okadaic acid treated cells showed internalized fluorescence at 3.5 hr equal to control cells at 0 min, cell treated with endothall showed higher internalized fluorescence. C. Cells treated with Cyclosporin showed higher internaized fluorescence at 3.5 hr while FK506 treated cells showed internalized fluorescence less than that of cyclosporine treated cells but comparable to control cells at 0 min.

Chapter 4

Discussion

Group I mGluRs can be regulated via multiple mechanisms. They can undergo endocytosis via two different pathways- agonist-mediated and agonist independent. For some GPCRs Endocytosis is a major mechanism of desensitization. Group I mGluRs can be desensitized via phosphorylation dependent pathway using second messenger dependent protein kinases or via phosphorylation independent pathways by GRK2 and optineurin. The long-form group I mGluRs (mGluR1a, mGluR5a, and mGluR5b) have been identified with a unique C-terminal tail which have a conserved PP1γ1 binding motif that strongly suggests the possibility of protein-protein interactions and phosphorylation(61). Studies suggest that phosphorylation of group I mGluRs is regulated by protein kinases like PKC, CaMKII, and GRKs, and PPs also play a significant role in regulating phosphorylation processes of group I mGluRs. Phosphorylation is an important process in regulating the clustering, trafficking (internalization and externalization), anchoring, signaling, and turnover of the receptor (Liu et al., 2006).

The binding, phosphorylation and dephosphorylation of group I mGluRs and their roles in regulating receptor function and trafficking has not been thoroughly investigated. Thus here we have studied the effect of phosphatase inhibitors in recycling of constitutively endocytosed receptors mGluR5b. We have used dual labelling assay in the endocytosis and recycling experiments to compare surface/internal receptor expression taking into account the variability among cells. It also allowed us to label only the surface receptors while keeping the internal fluorescence at minimum.

We have first seen the constitutive endocytosis process in which the receptors show maximum internalization at 30 min. Then we have seen that the receptors recycle back to the surface at 3.5 hr. Previous study has shown that these receptors do not co-localize with Lamp1 which is lysosomal marker, but they colocalize with Rab11 which is a recycling endosome marker (74). Also, in the lab it has been observed that in case of ligand-mediated endocytosis of mGluR5b, okadaic acid and endothall block the recycling of the receptors suggesting a role of PP1 and PP2A in dephosphorylation of the receptors (personal communication with Prabhat Mahato). Thus we investigated whether during the recycling process phosphatases are involved in resensitization of the constitutively internalized mGluR5b receptors. We used okadaic acid and endothall for inhibiting PP1 and PP2A. Okadaic acid is a weak inhibitor thus at low concentration it inhibits only PP2A while it

inhibits both PP1 and PP2A at higher concentrations. Endothall on the other hand inhibits both PP1 and PP2A. The first experiment showed no blocking of recycling of constitutively endocytosed receptors by okadaic acid or endothall, while blocking of endocytosis by cyclosporin and FK506. However in the second set of experiments, Okadaic acid showed no effect on recycling whereas endothall showed full block of recycling process. Since both the inhibitors act on the same phosphatases this result cannot be concluded and needs further investigation.

Both cyclosporin and FK506 on the other hand inhibits PP2B. However in the experiment with respect to the control cells while cyclosporin showed blocking of recycling subsequent to constitutive endocytosis of mGluR5b, FK506 did not block the recycling of the receptor. This is also inconclusive. Moreover the control cells did not show a full recycling of the receptors.

The laser light for 561nm of confocal microscope was at its last stage of its lifetime thus was fluctuating while the other laser light was steady. This might have caused some anomaly in the intensity of the receptors tagged with Alexa 568 which could affect the ratio of intensity of surface and internalized receptors. Secondly, the reason for contradictory result may be because the experiment did not work properly. Thereby more research needs to be done to come to any conclusion.

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